

AWARD NUMBER: W81XWH-14-1-0182

TITLE: Impact of the Ovarian Microenvironment on Serous Cancer

PRINCIPAL INVESTIGATOR: Joanna Burdette

CONTRACTING ORGANIZATION: University of Illinois at Chicago
Chicago, IL 60612-4305

REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5014

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2016		2. REPORT TYPE Annual		3. DATES COVERED 1Aug2015 - 31Jul2016	
4. TITLE AND SUBTITLE Impact of the Ovarian Microenvironment on Serous Cancer				5a. CONTRACT NUMBER W81XWH-14-1-0182	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Joanna E. Burdette E-Mail:joannab@uic.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Illinois at Chicago 809 S Marshfield, Rm 520 Chicago, IL 60612-4305				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command 820 Chandler Street Fort Detrick, MD 21702-5014				10. SPONSOR/MONITOR'S ACRONYM(S) USAMRAA	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Many now believe that the fallopian tube epithelium is the progenitor cell type for high-grade serous ovarian carcinoma. The initial phase of tubal cell metastasis could involve several sets of molecules, each of which represents a possible therapeutic target for intervention that would block serous cancer while still confined to the fallopian tubes. Using a series of normal, modified, and tumorigenic tubal cell lines, we will investigate the properties that allow tubal cells to migrate and adhere to novel three-dimensional ovarian organ cultures. We will also determine if the ovarian factors are necessary to fully transform fallopian tube cells. Using these as experimental models of pathway-modified or tumorigenic cells of tubal origin, we will investigate if ovarian factors enhance migration as a mechanism to explain the presence of tumor mass in the ovary of serous patients. 3D ovarian organ culture conditioned medium will be used as the chemoattractant. Collagen is a well-established matrix utilized by serous cancer cells, of unknown origin, to seed metastatic sites, such as the mesothelium. An RNAseq analysis will be performed between human TEC adhered to collagen matrix compared to tissue culture plastic and used to identify gene expression changes responsible for adhesion on collagen. Ovarian conditioned medium (OCM) with and without H ₂ O ₂ treatment will be added to normal and our series of pathway-modified oviductal cell lines to determine if proliferation and growth in soft agar are enhanced by factors in the OCM.					
15. SUBJECT TERMS Nothing listed					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 13	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION: The goal of this grant proposal is to understand how the ovary contributes to the migration, adhesion, and transformation of serous tumors that are derived from the fallopian tube. The initial phase of tubal cell metastasis could involve several sets of molecules, each of which represents a possible therapeutic target for intervention that would block serous cancer while still confined to the fallopian tubes. Using a series of normal, mutated, and tumorigenic tubal cell lines, we have investigated the properties that allow tubal cells to migrate to novel three-dimensional ovarian organ cultures, adhere to 3D collagen surfaces, and respond by proliferating to secreted ovarian factors.

BODY: We have made significant progress and achieved the major deliverables for year 2 of our proposed research. As outlined in our statement of work, our proposal had three aims. The first aim was to determine if secreted factors enhance migration of tubal epithelial cells. Our preliminary data had identified IP 10 (CXCL10), TIMP1, KC, MCS-F, and CCL2 (also known as MCP1). In addition, a paper was published suggesting that stem-like cells isolated from a high grade serous cancer patient of unknown cellular origin was attracted to the ovary because of SDF-1¹. These were all tested on the normal murine oviductal cells (MOE) and none of them significantly increased migration (data not shown). Since this result was a surprise, and not what we anticipated, we next re-examined ovary conditioned medium and confirmed that it enhanced fallopian tube migration (Year 1 report). This time we also analyzed fallopian tube conditioned media. We found ovary conditioned media increased migration 4x more than fallopian tube conditioned media, evidencing a unique role for the ovary (data not shown). Size fraction, heat inactivation, and proteinase K treatment all indicated the migratory factor to be a protein. Thus

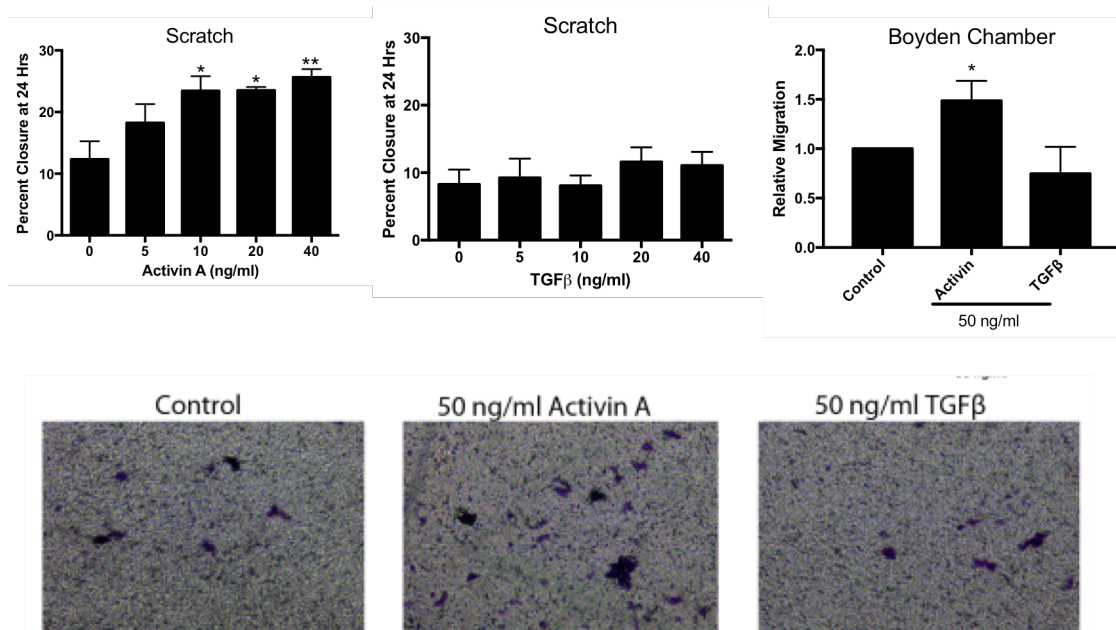


Figure 1. Activin, a secreted hormone from the ovary, stimulates fallopian tube motility and migration.

we evaluated the >3kDa molecular weight fraction by LC-MS. We found more than 600 proteins in the conditioned medium. The protein known as Activin A immediately was of interest because activin is a known ovarian secreted peptide hormone produced by the dominant follicles during follicular maturation. We confirmed that activin was able to significantly increase the motility of fallopian tube cells using a wound closure assay and Boyden chamber assay. We also tested

TGF β 1 (not present in ovary conditioned media) was a control, as it is in the same superfamily and activates the same intracellular signaling transcription factors (Smads2 and 3) through different receptors. Interestingly, TGF β 1 was not able to stimulate the same migration. We further validated this in a Boyden chamber migration assay (Figure 1).

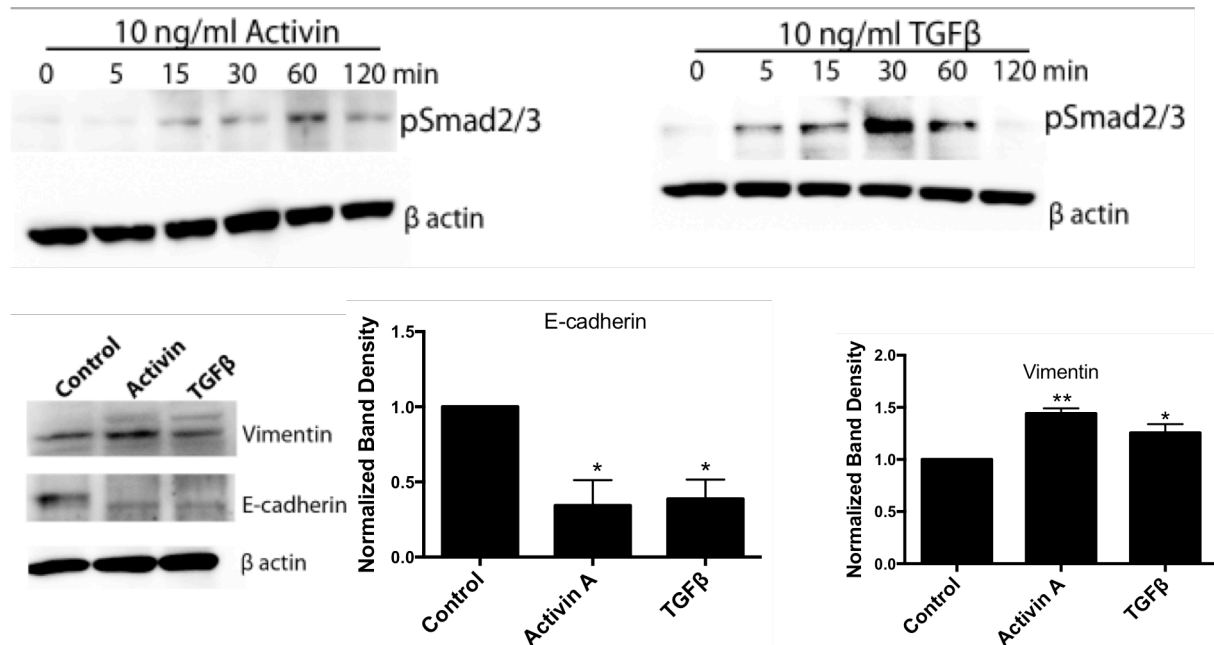


Figure 2. Activin stimulates Smads to modify EMT markers, vimentin and E-cadherin, consistent with increased invasion and motility.

We next investigated the signaling pathway and mechanisms for how activin would change cell motility and invasion. Activin stimulated time-dependent phosphorylation of Smad2/3 and so did TGF β 1. In addition both activin and TGF β 1 significantly increased the epithelial-mesenchymal marker vimentin and repressed E-cadherin. Since both activin and TGF β 1 were both able to activate p-Smad signaling, but only activin resulted in significant changes in motility and migration, we

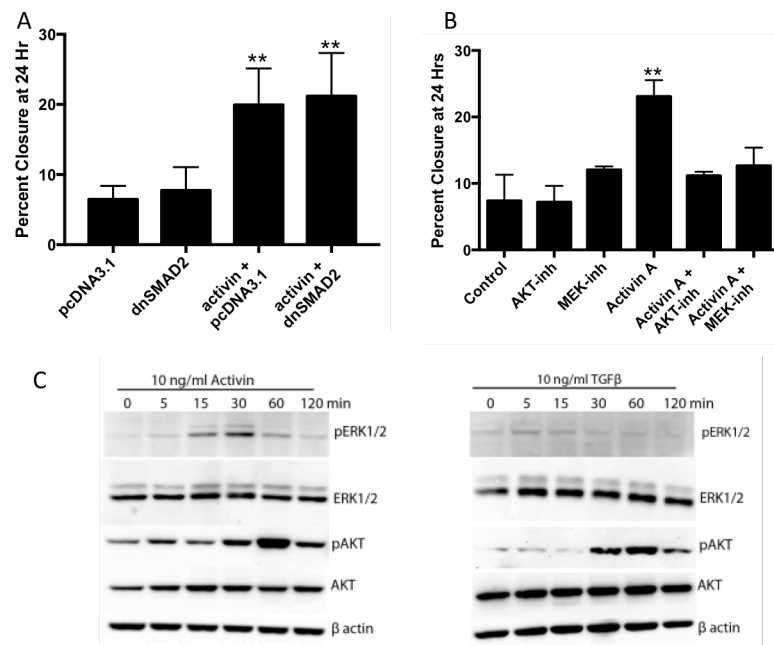


Figure 3. Activin stimulates Smads, Erk, and Akt.

investigated non-conical signaling pathways downstream of activin. First, we used a dominant negative Smad construct and demonstrate that when Smads are blocked, activin was still able to enhance wound closure of FTE (Figure 3A). Next, we investigated inhibitors of Akt and ERK1/2 (also called a MEK inhibitor) to see if they were able to block activin-induced migration and they were both able to significantly repress migration. We confirmed that activin was stimulated both p-AKT and p-ERK1/2. TGF β 1 also increased pAKT signaling, but did not enhance pERK1/2, suggesting that the change in migration is due to the unique activin stimulation of ERK (Figure 3C).

Next, we investigated the pathways downstream of ERK that were regulated by activin. We looked at Cdc42, RhoA, and Rac1. Only Rac1 inhibitor blocked the migration induced by activin. We then confirmed this using an siRNA directed against Rac1 (Figure 4).

Little has been reported regarding activin signaling in the fallopian tube, where HGSC tumors likely originate and then migrate to the ovary. We had a series of fixed tissue samples previously collected from CD1 mice that had been superovulated and compared to controls. We stained these already existing samples for phospho-Smad2/3 an indicators of activin signaling. We found that while the ovarian surface had activated Smad signaling both with and without ovulation, the fallopian tube had much more intense phosphorylated Smad staining after ovulation (Figure 5A). Because ovulation is associated with an increase in ovarian cancer risk and activin is produced by the ovary during folliculogenesis just before ovulation, this helps to connect the process of ovulation with fallopian tube stimulation that could enhance migration. Once FTE cells are transformed, this could increase ovarian colonization, and the initial phase of metastasis. We then used Oncomine to investigate whether activin mRNA expression was connected with high grade serous cancer. Serous tumors had significantly more activin receptor expression (ACVR1B and ACVR2A) and less TGFB3 (also known as betaglycan- which blocks activin) and INHA as compared to the normal ovary

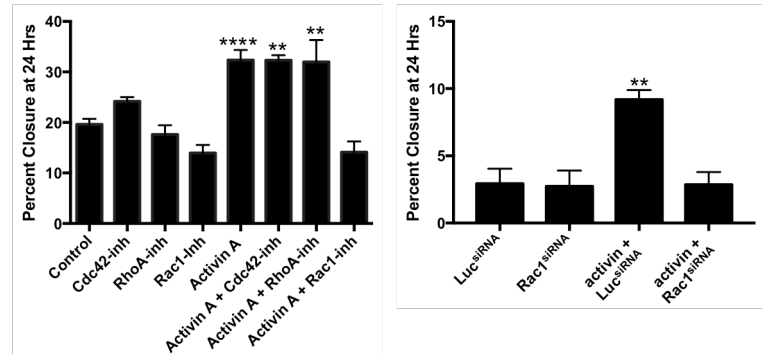


Figure 4. Activin regulated migration is through Rac1, which is downstream of ERK.

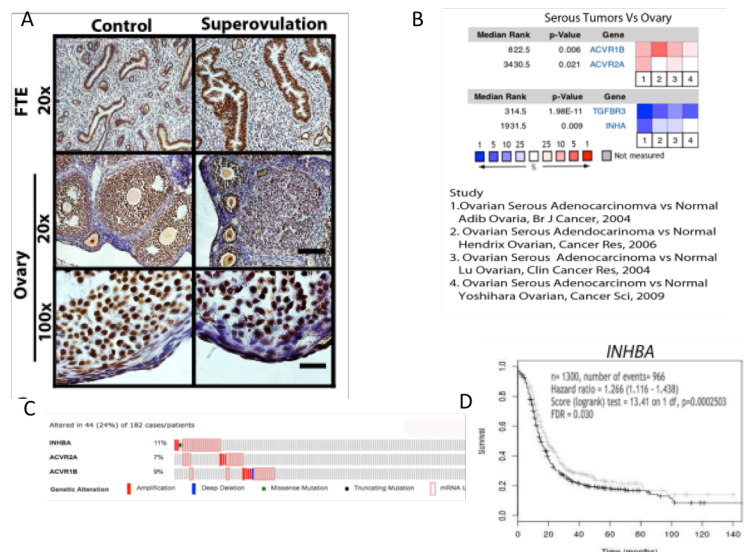


Figure 5. Ovulation increases signaling downstream of activin. Activin receptors are increased in tumors and associated with poor survival.

(Figure 5B). This is consistent with FTE derived cells that would have enhanced receptor to respond to the ligand being secreted by the ovary that drives metastasis. In the TCGA data set, these receptors were also enhanced in 16% of tumors. Activin (gene name INHBA) was increased in another 11% of tumors (Figure 5C). The online tool OvMark also indicated that patients with high activin expression had shorter survival times (Figure 5D).

Next we looked at two validated models of HGSC, OVCAR3 and OVCAR4. OVCAR3 responded to activin. Activin significantly and dose-dependently increased migration, which could be blocked with both ERK1/2 inhibitors as well as Rac1 inhibitors, similar to the biology seen in non-tumorigenic fallopian tube derived cell lines (Figure 6A-C).

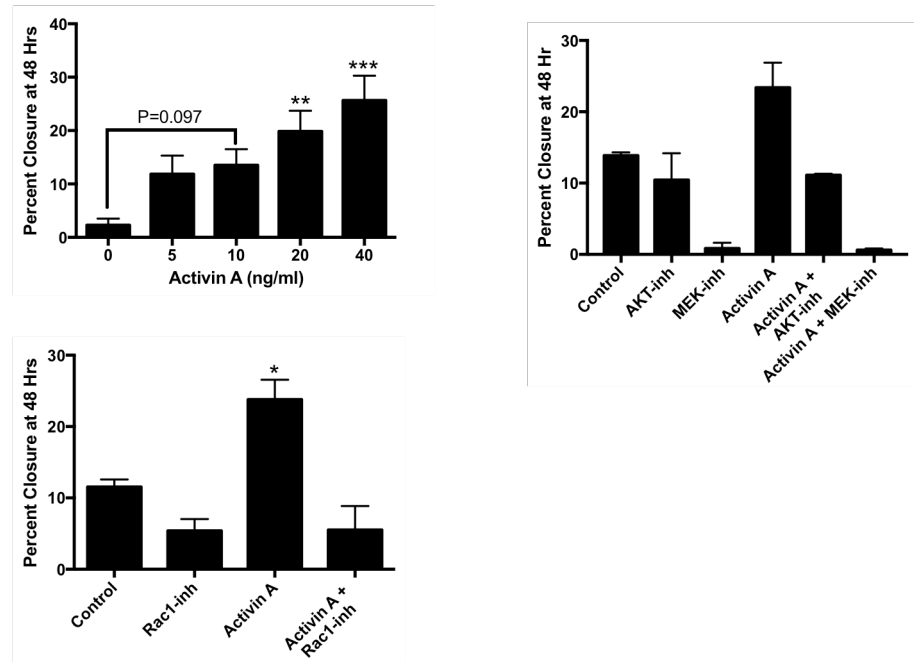


Figure 6. Activin increases migration of OVCAR3, which is mediated by ERK1/2 and Rac1, similar to FTE cells.

OVCAR4 when compared to OVCAR3 is already much more motile, and closes wounds more quickly. To confirm that these cells do migrate faster in response to common stimuli, they were cultured and wounded with and without fetal bovine serum, which is a common media supplement used to increase cell movement (Figure 7A). FBS increased migration in OVCAR4, but to a much smaller degree than in normal murine oviductal (fallopian equivalent in mouse) cells. Activin did not significantly increase migration except at the highest concentration (40 ng/mL)

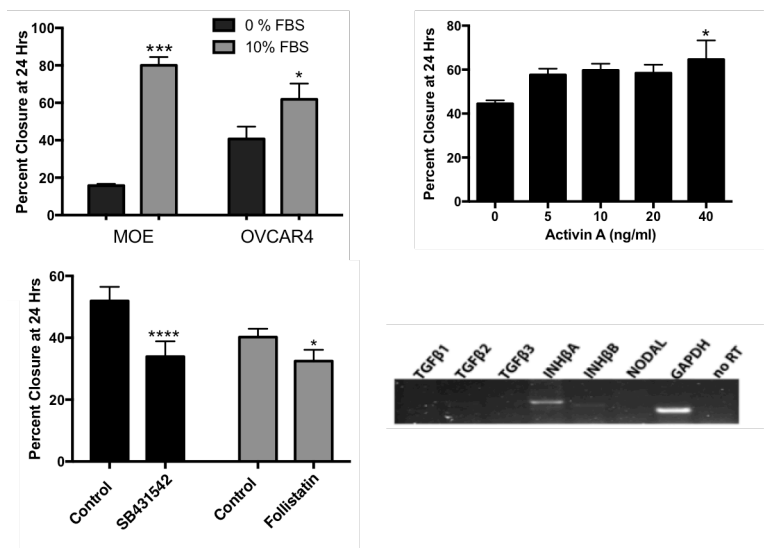


Figure 7. Activin increases migration of OVCAR4 and is produced and secreted by OVCAR4.

(Figure 7B). Both a small molecule inhibitor that blocks the Activin Type II receptor (SB413542) and a bioneutralizing protein (follistatin) were able to reduce migration in the absence of exogenous activin suggesting that the cells were making their own activin. PCR confirmed that OVCAR4 cells do make their own activin (gene symbol INHBA), which contributes to cell migration that can be blocked by antagonists. Lastly, we determined whether activin's stimulation of migration was due to activation of AKT, ERK, and Rac1. The Rac1 pathway seems essential for activin-induced migration in FTE, OVCAR3, and OVCAR4 (Figure 8).

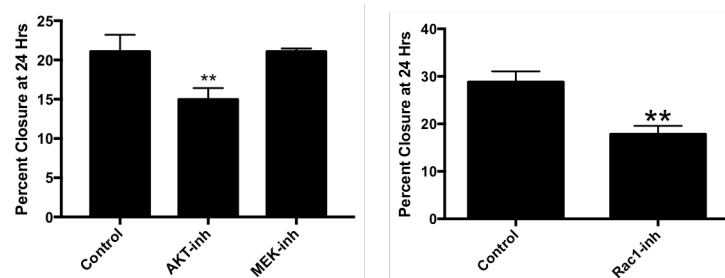


Figure 8. OVCAR4 are stimulated to migrate by RAC1.

To identify the migratory component in ovarian conditioned media, we have taken 2 approaches. First, size fraction revealed the migratory component to be between 50-100 kDa (larger than activin which is 26 kDa) (Figure 9A). Secondly, we tried numerous inhibitors in combination with ovarian conditioned media. Inhibition of autocrine motility factor (with E4P) did not block the

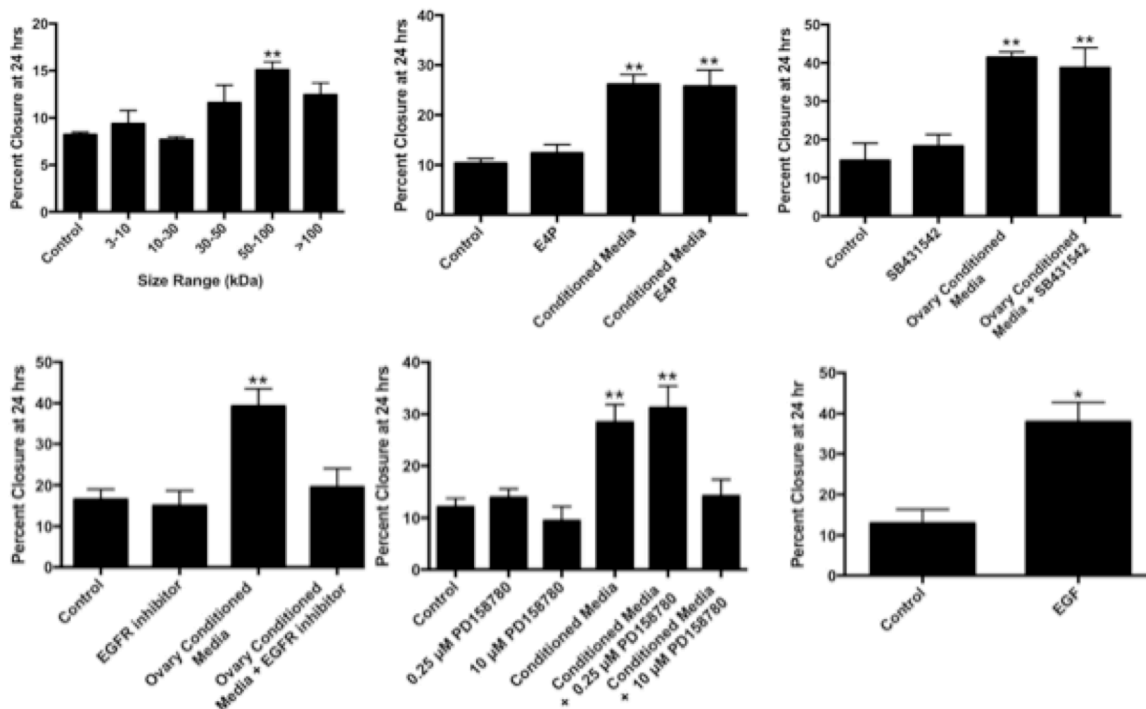


Figure 9. Conditioned medium contains a ligand between 50-100kDa that stimulates the EGFR to activate migration.

effect of conditioned media (Figure 9B). To test if activin A is the major migratory component of ovarian conditioned media, we treated conditioned media with SB431542, a TGF β /activin

inhibitor. However, SB431542 did not block the migratory effect (Figure 9C). The EGFR inhibitor completely abolished the effect of conditioned media (Figure 9D). PD158780 (an inhibitor with selectivity for HER2-4) was unable to inhibit the effect of ovary conditioned media at low concentrations (Figure 9E). At high concentrations of PD158780 (which would block EGFR and HER2-4) it blocked conditioned media's migratory effect (Figure 9E). Finally, EGF (not in conditioned media, used as a positive control) increased migration (Figure 9F). These results indicated a factor 50-100 kDa activated the EGFR to induce migration. Intriguingly there is no well-established EGFR ligand in the conditioned media. These results suggest EGFR is being activated by a novel ligand or is being activated indirectly. Future research will use fast-protein liquid chromatography (FPLC) to narrow down the list of proteins potentially stimulated migration.

For specific Aim 2, our focus was to monitor how cells adhere to 3D collagen as a mechanism for metastasizing to the ovary. In the previous report, we provided all of the data obtained regarding how to grow the cells on collagen, that they have a unique set of transcripts regulated by adhesion to collagen that revealed by RNAseq, and that the HGSC cells themselves survive better on collagen surfaces as compared to non-transformed normal FTE cells.

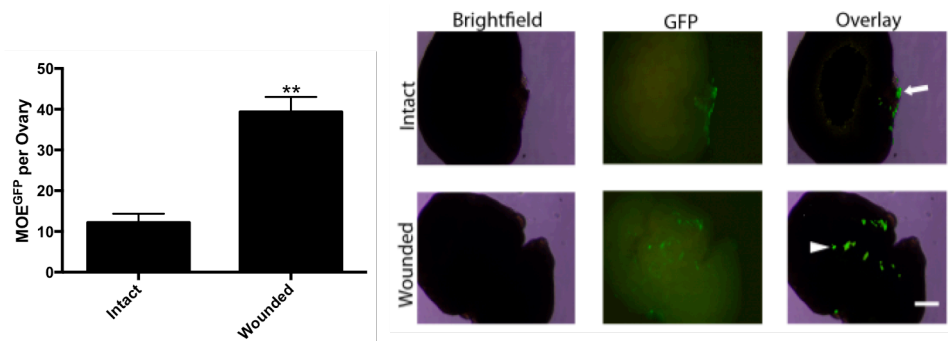


Figure 10. GFP labeled murine oviductal epithelium were co-cultured with murine ovaries with and without artificially wounded surfaced. Significantly more GFP-MOE were found to colonize the ovaries with wounded surfaces.

We speculated that FTE cells gain access to 3D collagen surfaces after ovulation and that this increases colonization. In order to better mimic this process, we used our 3D ovary culture developed from a previous DOD Pilot Grant Award. Using this system with GFP labeled FTE cells, we demonstrate that FTE cells better colonize ovaries with wounded areas that mimic ovulation (Figure 10). Ovaries express abundant collagen Type I in the areas surrounding

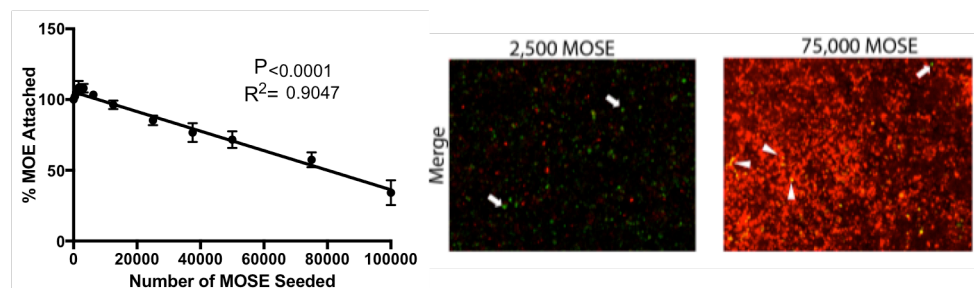


Figure 11. MOE cells adhesion decreases when MOSE cells are present suggesting they prefer to colonize exposed, wounded surfaces.

follicles. We cultured MOE cells on a variety of ECM and found that they adhere in short term assays equally well to type I collagen (a stromal ECM protein) as type IV collagen, fibronectin, and laminin (basement membrane proteins, data not shown). Next, we determined whether or not MOE cells prefer to adhere to ovarian surface cells or if they prefer to colonize areas devoid of OSE. By plating increasing numbers of MOE cells and MOSE cells on 2D collagen, we found that increasing numbers of MOSE cells results in significantly lower adhesion of MOE cells. We fluorescently tagged the cells and found that MOE cells prefer to colonize areas devoid of OSE cells as indicated by a lack of co-localized dyes (red + green = yellow). Very few green MOE cells are seen colocalized with red MOSE cells when a monolayer (75,000 MOSE) are plated (Figure 11).

We found that normal cells (MOE, MOSE, and IOSE80) had lower viability on 3D collagen relative to 2D. In contrast, the viability of HGSOC cell lines (OVCAR3, OVCAR4, OVCAR5, and OVASHO) was unaffected by 3D collagen (Data not shown), thus we sought to identify genetic alterations that enable cells to grow on 3D collagen using isogenic MOE cells produced as part of our previous DoD grant. Our RNAseq (year 1) identified increase p53 and PI3K/MAPK signaling pathways to be altered by culture on 3D collagen. We found that while expression of mutant p53 did not change viability of cells on 3D collagen, reduced PTEN expression enhanced viability on collagen (Figure 12). Our MOE models with PTEN^{shRNA} are the most aggressive in vivo and this may be due to their heightened ability to grow on collagen surfaces.

We then investigated if any of the transcripts regulated by PTEN^{shRNA} are regulated in the opposite direction of collagen to find candidate targets that govern this adhesion/growth mechanism. Several Adamts were regulated by 3D collagen and PTEN in opposite directions and we will now determine if PTEN^{shRNA} enhanced MMP15, Adam8, and Adamts4 provide an advantage for MOE cells to colonize 3D collagen surfaces and therefore metastasis to these sites, such as the wounded, OSE devoid ovary.

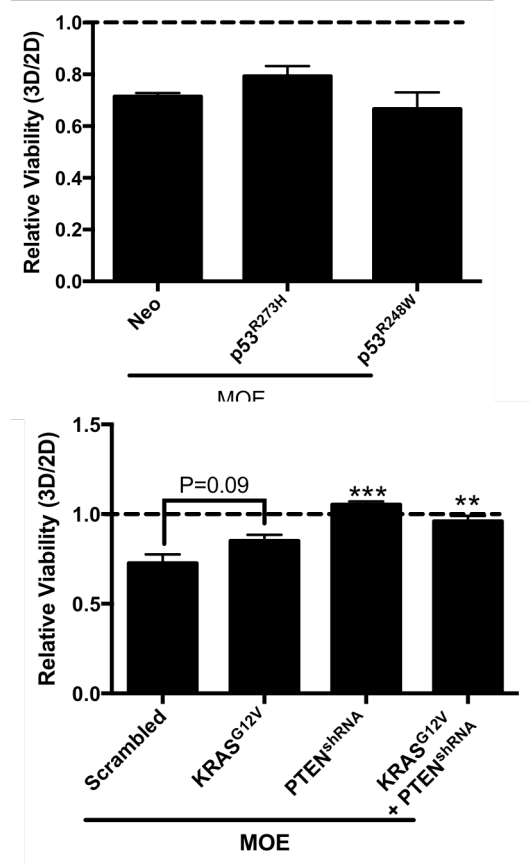


Figure 12. PTEN^{shRNA} improves growth on 3D collagen.

ECM Remodeling

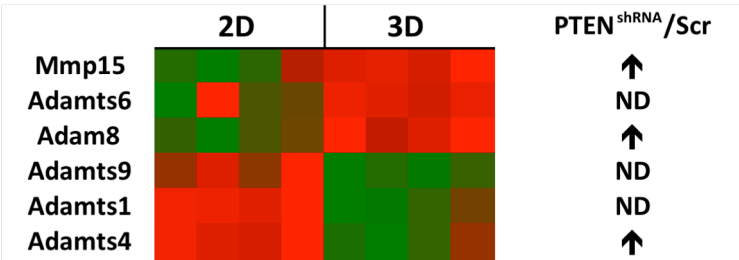


Figure 13. Genes regulated oppositely by adhesion to 3D collagen and PTEN^{shRNA}.

KEY RESEARCH ACCOMPLISHMENTS:

- Cytokines were not pro-migratory, but a protein constituent was confirmed to be responsible and mass spectrometry based proteomics and size exclusion chromatography are being used to purify and identify the active protein(s).
- Activin A (though not the major migratory component of ovary conditioned media) is secreted by the ovary and stimulates migration of FTE cells via a AKT/ERK/RAC1 pathway.
- A yet unidentified protein in ovary conditioned media stimulates migration via EGFR.
- Normal cells have reduced viability on 3D collagen, while HGSOc grow as well on 3D collagen as 2D collagen.
- RNAseq indicated that the top KEGG pathways modified by p53 signaling, adhesion, cytoskeleton, and MAPK signaling.
- Expression of mutant p53 did not rescue the viability of fallopian tube cells grown on 3D collagen, but knockdown of PTEN allows fallopian tube cells to grown on 3D collagen.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Abstracts and presentations

- Dean M, Canestrari E, David D, Burdette JE. (2016) Ovarian Secretome Profiling Identifies Proteins that Stimulate Migration of the Fallopian Tube Epithelium: A Mechanism for Initial Metastasis of High Grade Serous Cancer. 49th Annual Meeting for the Society for the Study of Reproduction National Meeting, San Diego, CA.
 - * Lalor Foundation Merit Award
- Dean M, David D, and Burdette JE. (2016) Secreted Proteins from the Ovary Stimulate Migration of the Fallopian Tube Epithelium: A Mechanism for Initial Metastasis of High Grade Serous Cancer. 3rd Annual Women's Health Research Day. University of Illinois at Chicago. Chicago, IL.
- Characterization of the Ovarian Secretome Identifies Proteins that Stimulate Migration of the Fallopian Tube Epithelium. (2015) Center for Biomolecular Science, University of Illinois at Chicago. Chicago, IL.
- Dean M, David D, and Burdette JE. (2016) Secreted Proteins from the Ovary Stimulate Migration of the Fallopian Tube Epithelium: A Mechanism for Initial Metastasis of High Grade Serous Cancer. College of Pharmacy Research Day. University of Illinois at Chicago. Chicago, IL.
- Dean M, Burdette JE. (2015) The role of the ovary in high grade serous cancer originating in the Fallopian tube epithelium. 7th Annual Illinois Symposium on Reproductive Sciences. University of Illinois Urbana-Champaign. Champaign, IL.
- Shaikh A, Dean M, Burdette JE. (2015) The role of cytokines in mediating migration of oviductal cells. University of Illinois Undergraduate Research Day: Posters Under the Dome. University of Illinois at Chicago. Chicago, IL.
- **degrees obtained that are supported by this award**
- **development of cell lines, tissue or serum repositories**
- **funding applied for based on work supported by this award**

CONCLUSION: Our results show that activin stimulates migration of tumorigenic fallopian tube epithelial cells toward the ovary through non-conical pathways. Also a yet unidentified protein that activates EGFR is the primary factor driving fallopian tube migration towards the ovary. We revealed that normal cells grow poorly on 3D collagen while HGSOC cells are unaffected. Cell lines from our previous DoD grant, we identified loss of PTEN as a key event in allowing fallopian tube derived cells to flourish on 3D collagen, and enhance colonization of the ovarian stroma. Comparing transcriptome profiles of normal fallopian tube cells grown on 3D collagen and PTEN^{shRNA} cells has highlighted MMP15 and ADAM8 as protein key proteins in adherence to and growth on 3D collagen. Lastly, while we hypothesized that the ovarian microenvironment would increase proliferation of fallopian tube cells, the secreted factors did not increase proliferation more than oviductal conditioned medium. This suggests that either additional mutations are required for fallopian tube cells to benefit from the ovary by proliferating or that the ovary may change as it ages to facilitate this biology.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

- 1 Yang-Hartwich, Y., Gurra-Soteras, M., Sumi, N., Joo, W. D., Holmberg, J. C., Craveiro, V., Alvero, A. B. & Mor, G. Ovulation and extra-ovarian origin of ovarian cancer. *Scientific reports* **4**, 6116, doi:10.1038/srep06116 (2014).

APPENDICES: N/A